



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68	A1	(11) International Publication Number: WO 95/03430 (43) International Publication Date: 2 February 1995 (02.02.95)
<p>(21) International Application Number: PCT/US94/08307</p> <p>(22) International Filing Date: 20 July 1994 (20.07.94)</p> <p>(30) Priority Data: 08/097,262 23 July 1993 (23.07.93) US</p> <p>(71) Applicant: GEN-PROBE INCORPORATED [US/US]; 9880 Campus Point Drive, San Diego, CA 92121 (US).</p> <p>(72) Inventors: RYDER, Thomas, B.; 1863 Angeles Glen, Escondido, CA 92029 (US). BILLYARD, Elizabeth, R.; 7512 Aegean Court, San Diego, CA 92126 (US). DATTAGUPTA, Nanibhushan; 4221 Kerwood Court, San Diego, CA 92130 (US).</p> <p>(74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon, 34th floor, 611 West Sixth Street, Los Angeles, CA 90017 (US).</p>	<p>(81) Designated States: AU, CA, JP, KR.</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>	
(54) Title: METHODS FOR ENHANCING NUCLEIC ACID AMPLIFICATION		
<p>(57) Abstract</p> <p>A method for amplification of a nucleic acid strand in a test sample. The method includes contacting the nucleic acid strand from the test sample simultaneously with at least three oligonucleotide primers. At least one primer is a promoter-primer, and at least one other primer is complementary to the nucleic acid strand, and one other primer is complementary to a strand complementary to the nucleic acid strand. The method further includes contacting the nucleic acid strand and primers with one or more proteins having RNA-directed and/or DNA-directed DNA polymerase activities, an RNA polymerase activity, and an RNase H activity under primer-extension conditions to allow amplification of a target region in the nucleic acid strand at essentially constant temperature.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

DESCRIPTION

Methods for Enhancing Nucleic Acid Amplification

This invention relates to amplification of nucleic acid strands using a DNA polymerase and an RNA polymerase at essentially constant temperature.

Background of the Invention

5 The ability to detect specific nucleic acid sequences has afforded many practical benefits in areas such as genetic research, clinical diagnostic testing, forensic sciences, archaeology, etc. In many cases, the sequence of interest might be present at a level much too low to
10 detect directly, even using probes with very high specific activity labels. In recent years, strategies have been devised for efficiently generating new copies of target sequences, including very powerful exponential amplification methods, which make it easier to accurately
15 detect the presence of very low target levels.

One such method is the polymerase chain reaction (Mullis et al., U.S. Patent 4,683,202) in which a reaction mix of primers, substrates, DNA polymerase and analyte nucleic acid is subjected to n cycles of heating to a
20 temperature sufficient for denaturing double-stranded nucleic acids and cooling to a temperature at which primer annealing and extension can occur. This reaction is well understood to have a maximum amplification factor of 2^n since each strand of a target sequence can be copied into
25 (at most) one new complementary strand during each cycle.

The performance of target-specific amplification has been augmented by performing two or more successive amplification reactions in which the target region defined by the primers used in the subsequent rounds is contained
30 within the target amplicon generated by primers used in the previous round. Even if the amplification of a desired target is inefficient in the first round because

of co-amplification of non-target sequences, the target amplicons that are generated should have a selective advantage for further amplification by the next primer set since non-target amplicons are usually not more effective
5 templates for further amplification by the nested primer set than other non-target sequences present. This strategy has been used to improve the ability of amplification methods such as PCR (Conway et al., J. Acquired Immune Def. Syndromes 3:1059 (1990); Matsumoto et al., J. Virol.
10 64:5290 (1990); Garson et al., Lancet 336:1022 (1990); and NASBA (Kievits et al., J. Virol. Methods 35:273 (1991)) to detect very low target levels.

The amplification method of Kacian et al. PCT-US90/03907 depends on multiple enzyme activities (*i.e.*,
15 including RNA polymerase, DNA-directed DNA polymerase, RNA-directed DNA polymerase, and RNase H). Although it is possible to provide these activities by contacting the other reactants with separate enzymes possessing one each of these activities, a preferred configuration uses a
20 single enzyme, reverse transcriptase, as the principal source of the last three activities listed above. For example, one embodiment of this method employs RNA polymerase from coliphage T7 and reverse transcriptase from Moloney murine leukemia virus (MuLV) in a reaction which
25 supports amplification extents of up to 10^{12} fold or more.

The rate of accumulation of products is much more complicated for such an asynchronous, continuous amplification process but is calculable based on straightforward physical properties of the reaction components.

30 The exponential accumulation of amplification products does not proceed indefinitely in any of these methods. The rate per time of new copy production reaches a maximum as the enzymes present become saturated by the number of existing templates available to be copied.
35 Thus, the system changes with time to a linear, rather than exponential, rate of accumulation. Ultimately the amount of product made is limited by the number of mole-

cules of those substrates, such as primers and nucleosides, which are physically incorporated into amplification products.

Summary of the Invention

5 This invention relates to a significant improvement of the process described by Kacian et al. In particular, it relates to methods for improving the sensitivity of the process, i.e., the ability to amplify desired target sequences that are present in extremely small numbers.

10 Applicant believes that the most significant and prevalent obstacle to achieving maximum sensitivity is competition for reaction components by amplification of non-target sequences. Although primer annealing and extension should be most efficient on target sequences

15 which are highly complementary to the primer, the possibility that a primer can complex with, and be extended upon, a sequence with only a few bases of complementarity to the 3' end of a primer is thermodynamically predictable and empirically known in the art. Even if the frequency

20 per site of non-target initiation is low, the number of non-target bases in a reaction is usually much greater than the number of targeted bases complementary to the primers used to select the target sequence. Since a primer is physically incorporated into the initiation

25 product, subsequent complementary copies can be very active templates for further amplification even though the original progenitor sequence scarcely resembled the desired target.

 The relative specificity of initiation by different

30 primer sequences can vary over quite a great range and while the specificity cannot reliably be predicted based on sequence alone, it is possible to identify preferable sequences by routine experimentation. However, the considerations described above imply that for even the

35 best primers, the potential for interference by non-target initiation becomes increasingly severe as the number of

target molecules is reduced since it becomes more probable that at some point early in the reaction, the population of non-target amplicons will be larger than the population of target-specific amplicons. The difference between these population sizes can be amplified exponentially as the reaction proceeds, and it is possible in such a case that the depletion of reaction components by non-target amplification causes the reaction to slow or stop before the target-specific product reaches detectable levels.

It is well known in the art that the stability of a base-paired complex between two nucleic acid sequences decreases as the temperature is increased. This usually results in an apparent increase in the specificity of detectable hybridization since hybrid thermal stability depends on the extent and continuity of base-pairing. Improvements in the yield of target-specific amplicon and reduction in the accumulation of non-target products were observed when the availability of a thermostable DNA polymerase made it possible to use higher reaction temperatures for PCR (Saiki *et al.*, Science 239:487 (1988)). Flexibility in selecting a reaction temperature has simplified effective optimization of PCR systems by routine experimentation (Rychlik *et al.*, Nucleic Acids Res. 18:6409 (1990)). However, development of systems for reliable detection of very low target levels (*e.g.*, <50) remains challenging.

Although raising the temperature reduces the lifetime of base-paired complexes once formed, higher temperatures also increase the rate of collisions between molecules to form potentially extensible complexes. Applicant has found that the amount of non-target priming increased at temperatures both above and below a measured optimum. Thus, it is rare that one can expect to achieve absolute specificity for the desired target based on controlling the temperature alone.

Other strategies have been described for enhancing the specificity of primer extension including use of

chemical denaturants and single-stranded binding proteins. Although these strategies have been useful in some cases, consistently favorable conditions have not been described.

At this time, thermostable variants of reverse tran-
5 scriptase which retain all three activities noted above are not known. Thermostable RNA polymerases have been described but none as yet having a promoter specificity as well-characterized as T7 RNA polymerase. Methods are known in the art to screen for, select for, and/or engi-
10 neer enzyme variants with desirable properties, including thermostability, but the methods disclosed herein afford another solution to the challenge of enhancing initiation specificity and, consequently, the sensitivity of target amplification. These methods have been especially
15 effective in compositions having a small number of target sequences in the presence of a vast excess of non-target nucleic acids, and furthermore, can be employed together with elevated temperature treatments.

The methods disclosed herein employ the concept of
20 amplicon nesting, but are significantly different from previously described strategies in which a portion of a reaction run with the first primer set is transferred to a new reaction containing the second primer set. In the methods described herein, all the primers delimiting the
25 nested amplicons can be combined in a single reaction such that serial transfer of products to a new reaction is unnecessary, and furthermore, the best mode is apparently favored by a dynamic coordination among their activities.

Increasing the number and types of primers present in
30 the mixture does significantly increase the potential for various side reactions, including those leading to competitive, non-target amplification. The extra primers added also have the potential to interfere with the desired normal function of the principal primer set.
35 Therefore, it was unexpected that we could identify conditions wherein the degree of enhancement was not only unequivocal but of such a dramatic extent. Note that the

method functions through a continuous process and does not require or employ any heat treatments to thermally denature double-stranded primer extension products.

Thus, in a first aspect, the invention features a method for amplification of a target sequence in a nucleic acid strand in a test sample. The method includes contacting the nucleic acid strand from the test sample simultaneously with at least three oligonucleotide primers. At least one primer is a promoter-primer (*i.e.*, having a primer region complementary to the nucleic acid strand or its complement, and another region, 5' of the primer region, recognized in its double-stranded form by an RNA polymerase), and at least one other primer is complementary to the nucleic acid strand, and one other primer is complementary to a strand complementary to the nucleic acid strand. The method further includes contacting the nucleic acid strand and primers with one or more proteins having RNA-directed and/or DNA-directed DNA polymerase activities, an RNA polymerase activity, and an RNase H activity under primer-extension conditions to allow amplification of a target region in the nucleic acid strand at essentially constant temperature.

A "test sample" includes any clinical, agricultural, or environmental sample which may or may not be pretreated to make the nucleic acid strand available for hybridization with the primers. Such a strand is not amplified by other methods prior to the first contacting step described herein. That is, the method of this invention can be used directly to amplify a nucleic acid within such a sample. No prior amplification by PCR or the method of Kacian *et al.* is necessary. The method essentially features the method of Kacian *et al.*, but with an additional primer provided to significantly and unexpectedly enhance target amplification at the expense of non-target amplification.

By "oligonucleotide" is meant to include a nucleic acid molecule with at least two nucleoside residues joined through a phosphodiester linkage, or an analog of a

phosphodiester linkage known in the art. The nucleotide base moiety of the oligonucleotide may be adenine, guanine, cytosine, thymine, uracil, or other naturally-occurring or synthetic base derivatives, especially those which can complex with a complementary base in another nucleic acid sequence to participate in a double-stranded nucleic acid structure. The sugar moiety may be ribose, deoxyribose, or other derivatives or modified forms of these structures. Many derivatives of the phosphodiester moiety are known in the art and can be used in the invention. An oligonucleotide may also contain domains or residues which are not nucleosides and which might be used, e.g., as a linker to a label or solid support, or to provide other functionality. Oligonucleotides can be synthesized chemically or by use of nucleic acid polymerases, or processed from naturally occurring nucleic acids, by many methods which are well known in the art.

By "primer" is meant a molecule which can be used by a nucleic acid polymerase as a receptor for covalent addition of a suitable nucleoside-5'-phosphoryl (or equivalent) residue. It is convenient to use an oligonucleotide with an extensible 3' end as a primer since it is straightforward to control the sequence of the primer and thus influence the polymerase to copy desired target sequences which are adjacent to sequences complementary to the primer; however, other molecules with priming activity, such as some proteins, are known.

By "promoter-primer" is meant a primer which also has sequence or structural properties which can interact with an RNA polymerase to cause the RNA polymerase to transcribe a desirable template. The promoter-primers used in the examples herein are oligonucleotides which consist of sequences known to be part of an effective promoter for T7 RNA polymerase linked to sequences which are complementary to desired targets in, e.g., the HIV genome. Other promoter sequences are known and can be used including promoters for T3 RNA polymerase and SP6 RNA polymerase.

Other strategies can also be employed to promote relatively specific transcription and are intended to be covered by this definition of promoter-primer. For example, an RNA oligonucleotide which is hybridized to a DNA template, especially in a heterotriplex structure (sometimes called an R-Loop) resembling a nascent RNA transcript, can be extended by an RNA polymerase to yield an RNA complement of a desired target template.

By "target region" or "amplification target" is intended to mean a sequence of consecutive nucleotide residues which one desires to amplify by duplication of this sequence or its complement by successive rounds of nucleic acid polymerization. It is not necessary to know the nucleotide sequence of the entire target region but it is helpful to know enough sequence to design at least one complementary primer and a sequence which can be used for specific detection of amplification products, such as by hybridization with a labeled complementary probe.

The phrase "non-target nucleic acid" includes all sequences which are not contained within such a desired target region. These might include, for example, other sequences present on the same genome as the target region, nucleic acids from other genomes or their gene products present in the reaction, such as from a host cell or from environmental contaminants, and nucleic acids deliberately added to the reaction, such as the primers.

In preferred embodiments, the nucleic acid strand is a single-stranded DNA strand or converted to single-strands by denaturing double-stranded DNA; the nucleic acid strand and primers are first contacted at 60°C or above with an enzyme having DNA polymerase activity active at 60°C or above; the second contacting step is at 42°C or above in the presence of a reverse transcriptase and an RNA polymerase; four primers are used in the first contacting step; at least one primer is provided at a concentration different from one other primer; all enzyme activities are provided by a reverse transcriptase and an

RNA polymerase; but its enzyme activities may be supplemented by an RNase H having no DNA polymerase activity; the DNA polymerase lacks 5'-3' exonuclease activity, and is derived from the DNA polymerase I of a Bacillus species; e.g.: of the species Bacillus stearothermophilus or Bacillus caldotenax; the two outside primers hybridize to said nucleic acid strand or its complement at most 2000, 500, or 350 bases apart; and one primer is provided at a concentration between 1 and 10 μ M and another said primer is provided at a concentration between 10 and 50 μ M.

In other preferred embodiments, two primers are plus-sense primers and the inside plus-sense primer is a promoter-primer; or two primers are minus-sense primers and the outside minus-sense primer is a promoter-primer. References to position and polarity are intended to have the meanings described below in reference to the structures in Fig. 1 and do not depend on polarity designations which might be conventional for the genetic system in which a target region is found. Thus, T74116 and 4195 in Fig. 1 are considered herein to be inside primers; T74312 and/or 4009 are considered to be outside primers. Of the possible amplicons which can result from this array of primers, it is expected that the sequence within the target region delimited by the inside primers will amplify to the greatest extent because amplification products which are delimited by one or both outside primers are targets for annealing by the complementary inside primer but the converse is not necessarily true. Therefore, the target region delimited by the inside primers in Fig. 1 is considered to be the principal target region, and T74116 is an example of the principal promoter-primer.

The sense of the endogenous target region which is complementary to the principal promoter-primer is defined as negative or minus sense, as are other nucleic acids present which have the same sequence sense as the minus target strand. Thus, the principal promoter-primer is

defined as positive or plus sense, as are other nucleic acids present that are complementary to the minus sense nucleic acids. It will be apparent to those skilled in the art that these assignments are valid even if the native form of the endogenous template containing the target region is a single-stranded nucleic acid molecule (e.g., RNA) since this strand comprises sufficient information to uniquely specify a complementary strand, and such a complement can be synthesized by the reaction components.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

FIG. 1 is a diagrammatic representation of the position of primers relative to the structure of the pol target region;

FIG. 2 is a diagrammatic representation of amplification Initiation Methods, IM1, IM2 and IM3 protocols.

FIG. 3 is a diagrammatic representation showing a possible scheme for initiation by extension of outside T7 primer; and

FIG. 4 is a diagrammatic representation showing potential strand displacement activity of T74116 primer extension product by subsequent extension of 4009 primer which may help make target-specific initiation more efficient.

Examples

The following are non-limiting examples of the present invention. Those in the art will recognize that variations to these examples are within the scope of the

appended claims. In particular the specific amounts of reagents and their proportions, and the specific enzymes and nucleic acids used, can be varied to allow amplification of any chosen target. In these examples, certain
5 terms are used as follows.

"Initiation" refers to the process by which an endogenous template sequence is copied or converted into a form which can be transcribed efficiently to yield RNA copies of the target region or its complement (whether
10 this is a desirable target region or not). In the amplification method of Kacian et al., initiation at a particular target is complete when such an RNA product is capable of participating as a template in a cycle of reaction steps, which can lead to de novo formation of a
15 template that can be transcribed to yield an essentially similar RNA molecule. (This RNA molecule may not be identical in sequence to its precursors but retains at least enough sequence similarity to be amplified further).

"Amplicon" refers to a nucleic acid that is a product
20 of one of the reactions in the amplification cycle and which retains the ability to serve as a template for further amplification.

"Pre-initiated template" is used to designate a nucleic acid that possesses the properties of an amplicon,
25 i.e., it can serve as a template for one of the reactions in the amplification cycle without first participating in one of the initiation reactions. A pre-initiated template may indeed be an amplicon product of a prior amplification reaction, or might be constructed synthetically as an
30 experimental model of amplicon activity by methods such as PCR, chemical synthesis or cloning.

As suggested above, the amplification reaction can be perceived as having two phases, one phase including those reaction steps causing the endogenous template to be
35 copied or converted into a functional amplicon, and the second phase including those steps that are part of the inherently cyclical amplification process. The intermedi-

ates and products of the amplification steps are essentially similar regardless of the original endogenous template, but the initiation steps used depend on the properties of the endogenous template. Various initiation strategies for the target amplification method of Kacian *et al.*, *supra*, have been described previously; some of them are described briefly here for convenience and shown diagrammatically in Fig. 2.

Referring to Fig. 2, Initiation Method 1 (IM1) refers to an initiation method in which the endogenous template is DNA. Under conditions allowing a promoter-primer to anneal to a complementary target, a DNA polymerase activity is added to synthesize a complement to the target template by extension of the promoter-primer. The reaction is heated (*e.g.*, at 95°C) to denature the double-stranded DNA product and cooled to a temperature which allows annealing of a second primer to a complementary sequence on the newly synthesized extension product. When suitable enzymes are added (*e.g.*, RNA polymerase, reverse transcriptase and, optionally, RNase H), the second primer can be extended by DNA polymerase activity to produce a double-stranded copy of the target region linked to a promoter, and thus an active template for the RNA polymerase.

Initiation Method 2 (IM2) refers to an initiation method in which the endogenous template is DNA. A single addition of enzymes (including RNA polymerase, reverse transcriptase and, optionally, RNase H) is sufficient to yield effective initiation even if the reaction is not heated to denature the initial primer extension products. Competent amplicons are generated in the reaction via intrinsic processes in the isothermal reaction.

Initiation Method 3 (IM3) refers to an initiation method in which the endogenous template is RNA. The reaction can be assembled and receive a single enzyme addition (including RNA polymerase, reverse transcriptase and, optionally, RNase H). The double-stranded product of

the initial extension of the promoter-primer is an RNA/DNA hybrid. The RNA strand is a substrate for the RNase H activity present and can be degraded to yield a single-stranded copy of the promoter linked to the target region, which in turn is a template for extension of the second primer as described above.

The terms "reaction failure" or "amplification failure" as used herein are not meant to imply that amplification failed to occur but simply that copies of the desired target sequence were not detectable among the products. This may indicate the absence of the desired target among the analyte nucleic acids. This might also result from target-specific initiation or amplification which was not sufficiently effective. For example, target-specific initiation might be ineffective even though many specific initiation events occurred if initiation on non-target sequences yielded excessive competitive amplicons. As will be shown in the examples below, the present invention provides sufficient improvement over existing methods to allow detection of as few as 1-5 copies of a target nucleic acid within a sample without requiring additional heating steps to denature reaction intermediates.

General Methods

The procedures described in this section, or slight variations thereof, were used in most of the examples described below. Exceptions and modifications are detailed in each example.

The following is an example of an IM2 amplification reaction.

- 1) A solution containing the following components was prepared and dispensed in a volume of 25 μ l:

14

200 mM Tris·HCl (pH 8.0 at about 20-25°C)
 70 mM MgCl₂
 8 mM spermidine
 0.4 mM deferoxamine mesylate
 25 mM each GTP & ATP
 10 mM each UTP & CTP
 0.8 mM each dATP, dGTP, dCTP, dTTP
 0.6 μM T74116 promoter-primer
 1.2 μM 4195 primer
 20% (v/v) glycerol

The primers used in the examples are shown diagrammatically in the figures. They have the following sequences:

SEQ.	ID	NO.	1	(4009):	5'-
'ATTCCCTACAATCCCCAAAGTCAA-3';					
SEQ.	ID	NO.	2	(T74116):	5'-
[AATTTAATACGACTCACTATAGGGAGA]CAAATGGCAGTATTCATCCACA-3';					
SEQ.	ID	NO.	3	(4195):	5'-
GTTTGTATGTCTGTTGCTATTAT-3'; and					
SEQ.	ID	NO.	4	(T74312):	5'-
[AATTTAATACGACTCACTATAGGGAGA]CCCTTCACCTTCCAGAG-3'. (The					

promoter sequences are shown in brackets, other promoter can be used in this invention.) The HIV sequences of T74116, 4195 and T74312 were disclosed previously (McDonough et al, U.S. Patent Application Serial No. 08/040,745 hereby incorporated by reference herein).

2) To this mixture was added 50 μl of a sample containing the nucleic acids to be analyzed. Model reference system samples contained 1 to 10 μg of purified human white blood cell (WBC) DNA in 80 mM potassium acetate. WBC DNA can be prepared by a variety of well-known methods (See, Maniatis et al., Molecular Cloning, a laboratory manual, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1982). Alternatively, 50 μl of a hydrolyzed WBC lysate, prepared as described in Example 4, was used. Reactions received 5 μl of water if negative controls, or 5 μl containing a known amount of purified,

cloned HIV nucleic acid for testing amplification performance.

- 3) The mixture was heated to 95°C and maintained at this temperature for 5 min. It was then transferred to 42°C and allowed to cool to this temperature.
- 4) Twenty μ l of a solution containing 800 U Moloney MuLV reverse transcriptase (RT) and 400 U T7 RNA polymerase was added in a solution comprising 50 mM Tris·HCl (pH 8.0), 10 mM potassium acetate, 100 mM N-acetyl-L-cysteine and 20% (v/v) glycerol.
- 5) This was mixed briefly and incubated at 42°C for 2 hr.
- 6) The formation of amplification product containing the intended target sequence was determined using a specific hybridization procedure. For all experiments described herein the hybridization protection assay (Arnold et al., Clin. Chem. 35:1588 (1989) and PCT/US88/03195) was used.

Unless specified otherwise in the examples below, the pol1 primers were used in the IM2 experiments at the concentrations listed above (i.e., 15 pmol T74116 and 30 pmol 4195 per 100 μ l reaction). When gag11 primers were used, T7811 and 872 were added at 30 pmol each per 100 μ l reaction.

Strategies for enhanced initiation effectiveness were tested using the following modifications of the basic IM2 procedure:

- 1a) A mixture of reaction components was prepared as described in Step 1 in the IM2 procedure. Optionally, additional oligonucleotides were added as outside primers, e.g., 3 pmol each per reaction of 4009 and T74312 for pol1 amplification.

- 2a) This mixture received 50 μ l of a sample containing the nucleic acids to be analyzed. Model reference system samples contained 1 to 10 μ g of purified human WBC DNA in 80 mM KOAc. Alternatively, 50 μ l of a hydrolyzed WBC lysate, prepared as described in Example 4, was used. Reactions received 5 μ l of water for negative controls, or 5 μ l containing a known amount of purified, cloned HIV nucleic acid.
- 3a) The mixture was heated to 95°C and maintained for 5 min. It was then transferred to 60°C and allowed to cool to this temperature.
- 4a) Optionally, 10 μ l of a solution containing a thermostable DNA polymerase was added in a solution comprising 50 mM Tris·HCl (pH 8.0), 10 mM potassium acetate, 100 mM N-acetyl-L-cysteine and 20% (v/v) glycerol. Enzymes tested and desirable properties thereof are described in the examples below.
- 5a) The reaction was mixed briefly and incubated at 60°C for 10 min.
- 6a) The reaction was transferred to 42°C and allowed to cool to this temperature.
- 7a) 10 μ l of a solution containing 800 U Moloney MuLV reverse transcriptase and 400 U T7 RNA polymerase was added in a solution comprising 50 mM Tris·HCl (pH 8.0), 10 mM potassium acetate, 100 mM N-acetyl-L-cysteine and 20% (v/v) glycerol.
- 8a) The reaction was mixed briefly and incubated at 42°C for 2 hr.
- 9a) The formation of amplification product containing the intended target sequence was determined using a specific hybridization

procedure such as the hybridization protection assay (Arnold et al., supra).

The outside primers used (if any) and their concentrations are described in each of the examples.

5 We found that the most valuable indicator of initiation effectiveness was the frequency of reaction failures for a particular template level rather than the extent of amplification in individual reactions. Therefore, for each condition tested, experiments were set up with
10 multiple replicate reactions so that improved initiation effectiveness could be identified by a statistically significant decrease in the failure frequency. Furthermore, the geometric means (G.M.) of the signals for the replicate reactions correlated well with initiation
15 effectiveness and are shown for most of the examples.

The HIV templates used in experiments described in the Examples were purified by standard methods from *Escherichia coli* containing plasmid clones of HIV sequences (see for example Maniatis et al., supra). In experiments specifying BH10 DNA, the template was a purified
20 double-stranded linear DNA having essentially the 8932 nucleotide sequence described in Genbank as HIVBH102 (Accession No. M15654) plus the complementary strand. Other experiments used a linearized plasmid DNA (pUCHIV)
25 comprising the gag and pol genes of BH10 in a standard pUC cloning vector. Both templates had virtually identical template activity per molecule in side by side comparisons.

After purification, the concentration of DNA in these
30 preparations was determined by measuring the amount of 260 nm ultra-violet light absorbed by samples of each preparation (A_{260}). The nucleotide sequence, and thus the length, of each of these DNA species is known. The molar concentration for such a preparation was determined by applying
35 standard conversion factors: mass concentration of double-stranded DNA = $50 \mu\text{g ml}^{-1} A_{260}^{-1}$; molecular weight of double-stranded DNA = $\text{length}(\text{bp}) \times 650 \text{ g mol}^{-1} \text{ bp}^{-1}$. A stock

solution of template DNA at a concentration $\geq 10^8$ templates per 5 μ l (33 pM) was divided into separate aliquots and frozen. For each amplification experiment an aliquot of template was thawed and serially diluted to the desired
5 working concentration (e.g., 5 templates per 5 μ l) for addition to reactions. The thawed aliquots and dilutions were discarded after each experiment.

Example 1: Initiation Effectiveness

To assess quantitatively the effect of various
10 reaction parameters on initiation effectiveness, it was desirable to develop methods to discriminate between changes in amplification effectiveness and in initiation effectiveness in response to a given variable. This was necessary because, for example, we found that conditions
15 favoring optimum amplification performance were not necessarily the conditions which yielded optimum initiation effectiveness. One way we accomplished this was to add pre-initiated templates to reactions as an indicator of the intrinsic amplification performance of various
20 reaction compositions or treatment scenarios and to compare these results with the amplification resulting from addition of a native target sequence.

Using this method, it was possible to determine how rapid and extensive the initiation of target-derived
25 amplicons must be to out-compete the amplification of non-target sequences. An amplification time course was performed in which reactions were assembled according to various desired test conditions but without any target template. At various times after the reaction was started
30 by addition of the RT and RNA polymerase enzymes, template was added and the resulting final amplification extents determined as described below:

- 1) A mixture was prepared containing the following
35 components, and 85 μ l of the solution was dispensed into each reaction tube. The concentra-

tions listed refer to the respective concentrations in the completed 100 μ l reaction.

50 mM Tris·HCl (pH 8.0 at room temperature)

- 5 17.5 mM MgCl₂
 5 mM dithiothreitol
 2 mM spermidine
 6.25 mM each GTP & ATP
 2.5 mM each UTP & CTP
10 0.2 mM each dATP, dGTP, dCTP, dTTP
 0.3 μ M each T74116 promoter-primer and
 4195 primer
 3 μ g human WBC DNA
- 2) The reactions were heated to 95°C for 7 min,
15 transferred to 37°C and allowed to cool to this
 temperature for 5 min.
- 3) Moloney MuLV reverse transcriptase (600 U) and
 T7 RNA polymerase (400 U) were added to each
 reaction in 10 μ l of buffer (10 mM Tris HCl (pH
20 8.0), 10 mM potassium acetate and 5 mM dithio-
 threitol).
- 4) At various times after enzyme addition, either
 100 copies of single-stranded BH10 DNA (puri-
 fied, cloned HIV DNA, previously denatured by
25 boiling) or 10 copies of pre-initiated template
 were added to respective reactions in a volume
 of 5 μ l. Three replicates of each time point
 and condition were processed.
- 5) After 2 hours the yield of target-specific
30 amplification product determined by the hybrid-
 ization protection assay (Arnold *et al.*, supra).

Analysis of the geometric mean of the signals of three replicates for each condition shows that even ten (10) pre-initiated amplicons could not be amplified to detectable levels if the amplification biochemistry is allowed to proceed for as few as 10 minutes in the presence of non-target nucleic acids but the absence of target

nucleic acid. Furthermore, about ten (10) times more endogenous template was required to achieve an essentially similar time course of amplification as was observed for the pre-initiated template. The difference is explained by the immediate entrance of pre-initiated template into the amplification cycle whereas the non-target amplicons already present continue to accumulate exponentially during the time required for the native template to be copied via the initiation reactions into an amplification competent form. For each of these conditions, more than 90% of the target-specific amplification potential was lost within 3-5 minutes of adding the reverse transcriptase and RNA polymerase to begin the amplification process. The extreme brevity of this window of opportunity for effective initiation was very surprising even though we had expected significant levels of non-target priming and initiation. The trends observed here, as well as in many comparable experiments, suggest that the inhibition was due to excessive depletion of essential reaction components by amplification originating from high levels of non-target initiation.

It has been possible to detect moderately low target levels in many cases using amplification systems developed by routine optimization of methods disclosed by Kacian *et al.*, *supra*. For example, using the IM2 method and the poll primer set we were able to detect virtually every test sample which contained ≥ 50 HIV genomes and about 2/3 of the samples containing 20 HIV genomes. This performance reflects very powerful amplification, which would be more than adequate for most purposes. There are, however, cases in which even greater sensitivity is desired. HIV is one example of a pathogen whose nucleic acids might be present at a very low concentration in infected tissues such as whole blood. Reliable detection of HIV nucleic acids sometimes requires a significant sample size (*e.g.*, WBCs from ≥ 0.1 - 1 ml blood or more) to ensure that at least one target sequence is present. The nucleic acid

extracted from such a sample might contain a single HIV genome in the presence of >20 μ g of non-target DNA. The severely aggressive character of competitive non-target amplification as revealed in Example 1 makes it clear that
5 detecting the target in such a sample was a very challenging goal and could not be expected by routine experimentation.

Example 2: Thermostable DNA Polymerase

This example demonstrates that significant increases
10 in sensitivity can be achieved by application of the principles disclosed here. Samples A and B, shown in Table 1 were treated using the standard IM2 method as described under General Methods above, i.e. the samples were cooled from 95°C directly to 42°C and the reverse
15 transcriptase/T7 RNA polymerase mixture was added to begin the reaction. Samples (C-F) were cooled from 95°C to 60°C, as described, received 2 U *B. stearothermophilus* (*Bst*) DNA polymerase each, and were allowed to incubate for 10 min. The samples were then allowed to cool to 42°C before
20 receiving the reverse transcriptase/T7 RNA polymerase mixture. Each reaction received purified cloned HIV DNA (pUCHIV) diluted to an average of 5 templates per reaction.

Table 1

Bst	Outside Primers (3 pmol)			
	None	T74312	4009	4009 + T74312
	A			B
None	2,822	n.d.	n.d.	5,575
	788,364			1,080,914
	5,609			598,645
	550,515			2,904
	54,499			399,264
	2,962			692,780
	884,319			3,057
	2,404			907,013
	5,601			3,386
	301,269			635,132
G.M.:	36,305			83,898
	C	D	E	F
2 U	2,684	894,475	996,174	1,053,438
	21,699	1,007,288	573,620	925,349
	500,660	10,027	933,090	985,495
	2,685	914,272	230,777	981,515
	222,122	897,114	982,900	953,186
	157,526	923,988	701,584	1,000,703
	518,992	942,281	802,113	1,011,202
	318,567	962,413	939,987	1,013,977
	736,861	963,703	3,605	958,185
	2,896	78,465	1,100,968	1,040,630
G.M.:	63,108	464,691	436,989	991,645

The amount of target sequence generated is expressed in Tables 1 - 7 in relative light units (RLUs), a measure of the amount of signal obtained from the chemiluminescent label on the detection probe.

The RLU values for the negative control (no pUCHIV) for each of these reaction conditions (A-F) were: 2591, 3097, 2471, 3569, 3459 and 6030, respectively.

These results show that using either a high temperature initiation step with Bst polymerase (C) or including the outside primers even at 42°C (B) can each alone enhance initiation effectiveness. The most dramatic

enhancements were seen when the 60°C supplemental initiation step was performed in the presence of either of the outside primers (D, E), and the best condition included both outside primers as well as the 60°C supplemental initiation using *Bst* DNA polymerase (F).

EXAMPLE 3: PRIMER TITRATION

This example shows some of the surprising properties of the enhanced initiation systems, which make it clear that these enhancements were not obvious nor predictable from prior art.

The most effective concentration of outside primers was determined by titration. In this example, both the T74312 promoter-primer and the 4009 primer were included at equimolar levels as shown in Table 2. The experiment was also intended to determine if initiation enhancement was due primarily to the primer nesting, to the high temperature step alone, to the high temperature incubation in the presence of DNA polymerase, or to some combination of these factors. The reaction condition (A), which had no *Bst* polymerase and no outside primers, was executed using a standard IM2 initiation as outlined under General Methods above (i.e., no 60°C step). All the other samples received the 60°C incubation step whether or not *Bst* polymerase was included in the reaction.

Each sample shown in the table received an average of 5 molecules of pUCHIV DNA. A negative control was also done for each reaction condition; the RLU values for the negative controls were: 1481, 3073, 1888, 1579, 2150, 1685, and 2038, for A-G, respectively.

Table 2

Bst	Amount of T74312 4009 &			
	None	0.5 pmol each	1 pmol each	3 pmol each
	A	B	C	D
None	1,539	2,613	1,839	3,196
	1,817	2,798	1,968	916,062
	276,389	2,618	1,859	71,336
	703,977	6,461	1,735	377,802
	504,437	2,499	1,827	322,609
	2,190	98,767	978,524	991,897
	112,011	2,563	1,767	932,431
	945,321	2,362	53,199	125,527
	450,767	17,165	1,713	716,442
	2,021	2,234	187,509	791,526
G.M.:	47,460	4,611	7,585	264,500
		E	F	G
2 U	n.d.	816,921	934,499	960,554
		2,405	925,259	920,915
		990,140	992,702	952,251
		990,692	979,840	1,012,172
		1,008,058	966,982	954,368
		957,396	997,355	1,011,579
		968,449	994,863	974,269
		957,421	982,283	1,008,390
		1,031,290	937,674	1,017,541
		2,055	934,387	1,023,782
G.M.:		285,949	946,198	982,999

As described above, it is possible for the extra primers included in the reaction to inhibit target-specific amplification by promoting additional initiation on non-target sequences. This potential for interference with desired amplification by extra primers in the reaction is observed here, *i.e.* in the reactions with 0.5 or 1 pmol each outside primer in the absence of the higher temperature pre-initiation step (B, C). In contrast, 3 pmol of each outside primer (D) yielded significantly better initiation effectiveness than the standard IM2

initiation condition (A). The inclusion of the 60°C primer extension step (E-G) not only broadens the range over which the nested primer strategy is effective, but also, as in the previous example, is synergistic with the best outside primer conditions (G) to yield impressive initiation effectiveness.

EXAMPLE 4: CRUDE LYSATES

This example showed that the initiation enhancements were not only functional, but even more valuable, when applied to a crude lysate typical of a patient sample after appropriate processing. Because of the complex and variable chemical composition of such lysates, it is typical for at least some of the amplification processes to proceed less effectively in lysate than in systems with purified components. Therefore, signals are often lower and/or failures more likely than for comparable target levels in a reaction containing purified components such as the model reference system reaction.

1) Whole blood treated with EDTA as an anticoagulant was mixed with an equal volume of a Density Centrifugation Medium (DCM) comprising PERCOLL in 0.25 M sucrose at a density of 1.110 g/ml. The mixture was centrifuged at 1600 X g for 20 min.

2) The mononuclear WBCs (MNC) were harvested by pipetting from a band that formed at the meniscus of the equilibrated mixture. The DCM/MNC suspension was mixed with an equal volume of 0.14 M KOH, mixed well and heated at 95°C for 30 min.

3) After cooling to room temperature, the resulting hydrolysate was adjusted to pH 8.0 ± 0.5 by adding one-tenth volume of a solution comprising:

0.65 N acetic acid

0.066 M Tris (hydroxymethyl) aminomethane
[Tris base]

0.084 M Tris·HCl

- 5 4) Amplification reactions received either 50 μ l of this lysate or 50 μ l of 1 μ g of purified human WBC DNA in 80 mM potassium acetate (Reference System).

Test reactions received the number of pUCHIV templates indicated in Table 3. One negative control reaction was done for each condition (A-D) and these values were: 2988, 2179, 5740, and 5602 RLU, respectively.

Table 3

	Standard	Enhanced
	A	B
Reference System 5 pUCHIV	12,849	992,207
	816,241	1,013,207
	10,397	987,214
	722,462	916,050
	478,359	1,004,124
	890,801	980,016
	615,661	951,094
	2,608	1,009,547
	605,710	988,996
	89,926	973,544
	C	D
Lysate 10 pUCHIV	5,381	844,281
	5,604	779,576
	5,568	888,957
	40,029	850,735
	4,980	905,316
	5,245	889,550
	5,385	611,966
	4,826	645,488
	4,937	849,922
	5,067	797,581

Although the reference system, standard IM2 results (A) showed good initiation effectiveness, it is evident that the enhanced system (B) is significantly better; all

10 signals are essentially saturated under these conditions. Furthermore, the lysate sample results (C, D) make it extremely clear how much benefit can be achieved from using the initiation enhancements.

5 EXAMPLE 5: OUTSIDE PRIMERS

This experiment re-examined the enhancement obtained with each of the outside primers under the challenging conditions of a low target level (3 PUCHIV) in the presence of lysate. All reactions received 1 U *Bst* DNA
10 polymerase and were subjected to the 10 minute incubation at 60°C. The outside primers used (at 3 pmol each per reaction) and the combinations tested are shown in the top row of Table 4. The primer 4312b has the identical sequence complementary to HIV as does T74312 but does not
15 have the promoter sequence.

The RLU values obtained from ten (10) replicate reactions for each condition are shown in Table 4. The bottom row of Table 4 shows the geometric mean of the individual replicate results for that condition. The
20 negative control results for each condition (A - E) were 776, 2850, 4053, 3875, and 4126 RLU, respectively.

Table 4

Outside Primers				
None	T74312	4312b	4312b & 4009	T74312 & 4009
849,178	866,790	929,644	824,030	716,145
3,867	804,203	3,506	4,451	910,595
382,761	4,064	959,438	829,668	880,340
374,433	901,779	895,765	861,299	726,859
284,409	896,920	3,950	240,405	922,937
43,293	850,814	3,892	960,310	866,289
3,893	883,606	3,637	944,199	992,987
3,722	1,083,540	867,171	941,293	925,316
27,165	998,443	858,293	895,293	875,782
893,489	920,823	930,880	909,846	958,355
67,752	528,996	100,820	461,481	873,055

As seen previously in Example 2, these results show that the outside promoter-primer T74312 can promote enhanced initiation even in the absence of 4009. Furthermore, these results strongly suggest that the enhancement potential of T74312 benefits from the promoter moiety since the homologous non-promoter-primer, 4312b, did not stimulate initiation significantly over the control condition with no outside primers. One possible mechanism that could account for these results is shown in Figure 3. It is likely that T74312 can initiate a IM2 process by being extended on its complement in the usual way. Note that this step should not interfere with the normal initiation steps primed by the principal promoter-primer, T74116, since they occur on different strands.

Initiation by T74312 in this reaction might be expected to be less efficient than by T74116 since T74312 is present at a lower concentration; however, any T74312 initiations that are successfully completed will result in multiple single-stranded RNAs, which are templates for highly efficient IM3-type initiation by T74116, and which can significantly and preferentially accelerate the accumulation of competent pol1 amplicons during the early stage of the reaction. It probably is desirable for the outside promoter-primer (e.g., T74312) to have lower activity in the reaction than the inside promoter-primer (e.g., T74116) since we have found that highly efficient transcription on both strands can inhibit effective amplification.

Note that different sequences can have different rates of hybridization to their respective complements even at identical concentrations. Therefore the ratio of priming activities for two different oligonucleotide sequences may not be the same as the ratio of their concentrations. However, the molar concentrations are a useful first approximation of the relative activities of two different promoter-primers, and the optimum ratio can be determined by routine experimentation. Furthermore,

methods for quantifying hybridization rates are well known in the art and can be used to resolve apparent anomalies in effective concentrations.

It is evident that 4009 improves initiation effectiveness in addition to any role it may serve in completing the formation of transcriptionally-active species initiated by extension of T74312 (diagrammed in Fig. 3). Not only did the presence of 4009 produce a clear enhancement in this experiment even when paired with the non-promoter-primer, 4312b, but it also promoted enhanced initiation in Example 2 in the absence of either 4312 species.

A possible enhancement mechanism consistent with these observations is shown in Fig. 4. Here, primer 4009 is capable of priming DNA synthesis, which can displace previously synthesized DNA extended from the inside promoter primer, T74116. It is desirable that this outside primer have lower activity than the inside promoter-primer to make it less likely that the outside primer will be extended first, rendering the primary target region double stranded and thus inaccessible to initiation by the inside promoter-primer (e.g., T74116).

EXAMPLE 6: DNA POLYMERASE PROPERTIES

The experiments shown in this example were done to determine if properties other than thermostability of the supplemental DNA polymerase were important to the initiation enhancement mechanisms. The results shown in Table 5 were from three different experiments, each with its own goals, but each contained similar controls which can be compared as references to judge the relative merit of each enzyme. The RLU values in the bottom group, labeled "None", were from standard IM2 reactions, incubated with no outside primers and no thermostable DNA polymerase. The middle group, labeled "Bst-1", was treated using the enhanced initiation procedure as described under General Methods and employed Bst DNA polymerase from Bio-Rad. The

top group shows the results of the same enhanced initiation procedure substituting one of the alternative thermostable DNA polymerases indicated in the column headings. "Bst-2" denotes a sample of Bst polymerase from a second vendor, Molecular Biology Resources; "Bca" corresponds to DNA polymerase from *Bacillus caldotenax* (TaKaRa); "REPLIT-HERM™" is a DNA polymerase available from Epicentre, "KLENTAQ™" DNA polymerase (Ab Peptides) is a derivative of *Thermusaquaticus* DNA polymerase as described below. The samples were all handled using the enhanced initiation methods described under General Methods. The respective DNA polymerases indicated were used for the 10 minute, 60°C incubation step. The reactions contained WBC lysates, or were the model reference system and received the average template inoculum shown in Table 5.

Table 5

	<i>Bst</i> -2	<i>Bca</i>	Replith- erm	KlenTaq
Reaction:	Lysate	Model Sys	Model Sys	Lysate
pUCHIV:	5	4	4	5
5 Test Enzyme	1,216,201	812,767	842,161	11,564
	1,041,976	801,084	818,499	811,042
	952,373	851,248	1,238	153,291
	1,039,396	855,734	866,246	566,063
	905,270	811,228	1,262	625,274
		846,270	865,195	427,127
		866,044	1,455	
G.M.:	1,025,760	834,568	52,998	245,204
<i>Bst</i> -1	547,626	944,662	944,662	906,711
	276,219	913,013	913,013	891,475
	710,203	906,523	906,523	654,163
	19,428	921,547	921,547	33,052
	728,553	954,490	954,490	813,337
		862,586	862,586	899,851
		891,032	891,032	
G.M.:	273,150	912,946	912,946	483,597
None	3,546	855,208	855,208	5,191
	3,207	1,259	1,259	5,029
	3,191	849,200	849,200	5,337
		1,334	1,334	5,417
		1,342	1,342	5,648
		1,277	1,277	5,579
		1,461	1,461	
10 G.M.:	3,311	8,441	8,441	5,363

Table 5 shows that several thermostable DNA polymerases other than *Bst* were also capable of supporting enhanced initiation effectiveness in concert with outside primers. In separate experiments, we found that some other thermostable DNA polymerases did not seem to act synergistically with the nested primers to yield enhanced initiation. These included native DNA polymerases from *Thermus aquaticus* (Taq), *Thermus flavus* (Tfl), *Thermus thermophilus* (Tth), *Thermococcus litoralis* (Vent™, New

England Biolabs), or Retrotherm™ (Epicentre). Some of these did confer improved initiation effectiveness compared to standard IM2 if used in an initial 10 minute, 60°C primer extension step in a reaction without outside
5 primers; however, this improvement was never as extensive as the fully enhanced system described above.

The four polymerase enzymes that did support fully enhanced initiation have at least one property in common, lack of a 5'→3' exonuclease activity which may contribute
10 to their effectiveness. *Bst*, *Bca* and *KLENTAQ* are each homologs of *E. coli* DNA polymerase I. The 5'→3' exonuclease that is usually found in this class of enzyme is removed by proteolysis during purification of *Bst* by both vendors. *Bca* and *KlenTaq* are both manufactured by expres-
15 sion from respective clones of mutant genes defective in this activity. *REPLITHERM* is reported by its manufacturer to lack any exonuclease activity. That the enhancement mechanism benefits from 5'→3' exonuclease deficiency is suggested here because of this correlation and further
20 corroborated by the superior efficacy of *KLENTAQ* compared to the native parent form of *Taq* polymerase.

In these and other experiments, the three polymerases from *Bacillus* species seemed to support more consistent, stable enhancement than either *REPLITHERM* or *KLENTAQ*;
25 therefore, these three related enzymes may share a property that distinguishes them from the other two. One possibility is that efficient strand displacement activity, which is known to differ among DNA polymerases, could contribute to mechanisms such as shown in Fig. 4, but
30 other possibilities are not excluded.

These insights showed that the benefits conferred by the fully-enhanced system were not simply dependent on a brief window of elevated primer annealing stringency enabled by the thermostable DNA polymerase, but that the
35 system as configured here has mechanistic advantages, which were not obvious, nor predictable, from prior art.

Example 7: Other Target Regions

The initiation enhancements were also tested and shown to work for target regions other than pol1. The inside target region shown here is called gag11 and uses the promoter-primer T7811 and the non-promoter primer 872. Primer placement for the nested gag11 target region corresponds to Fig. 1 with 780, T7811, 872, and T71062 substituted for 4009, T74116, 4195, and T74312, respectively. The sequences of these primers are:

10 SEQ. ID NO. 5 (780): 5'-TGCACCAGGCCAGATGAGAGAACCA-3'
SEQ. ID NO. 6 (T7811):
5'-[AATTTAATACGACTCACTATAGGGAGA]AGTGACATAGCAGGAACCTA-3'
SEQ. ID NO. 7 (872): 5'-AGATTTCTCCTACTGGGATAGGT-3'
SEQ. ID NO. 8 (T71062):
15 5'-[AATTTAATACGACTCACTATAGGGAGA]TTGGACCAGCAAGGTTTCTGTC-3'
where the bracketed sequence corresponds to the T7 promoter sequence as described previously (Kacian et al., supra). The promoter portion can be replaced with other functional promoter sequences as described above. The HIV
20 sequences of T7811 and 872 are disclosed in McDonough et al., supra.

In this example, 872 was used at 30 pmol/reaction. The principal promoter-primer, T7811, and the outside primers, T71062 and 780, were present at the indicated
25 concentrations. Otherwise, the reactions were handled as described under General Methods using 1 U of Bst DNA polymerase per reaction.

All reactions contained 50 μ l KOH-hydrolyzed lysate as described in Example 4, and positive reactions received
30 an average of 5 pUCHIV templates. Negative control results for each of these conditions (A - H) were 5682, 6501, 5775, 4954, 5689, 5140, 5079, and 4805 RLU, respectively.

Table 6

Principal Promoter Primer	Outside Primers (pmol/100 μ l)			
	780: 0	5	5	5
	T71062: 0	0	5	10
T7811	A	B	C	D
15	8,456	6,828	86,674	96,750
	14,254	5,873	197,119	51,773
	5,990	5,336	8885	61,521
	31,141	35,033	77,964	59,049
	18,771	14,259	76,564	41,677
	18,517	10,092	16,734	39,743
G.M.:	14,087	10,103	49,749	55,786
	E	F	G	H
30	20,083	20,020	91,414	119,865
	24,839	8,711	24,645	143,728
	6,795	15,840	36,989	75,950
	9,958	74,433	109,757	36,031
	5,980	19,589	39,540	77,141
	10,243	19,289	47,766	15,626
G.M.:	11,287	20,657	50,843	62,005

10 This example shows the benefits of titrating each primer independently. The results of this and other similar experiments are consistent with the expectation, as discussed above, that the optimum concentration of the outside primers should be lower than that of the inside primers. Further optimization improved the gag11 initiation enhancement even more as shown in the data in the next example.

EXAMPLE 8: MULTIPLEX AMPLIFICATION

20 In some cases it is desirable to amplify two or more distinct target regions in the same reaction. Such "multiplex" amplification reactions, containing 2 or more pairs of primer sets, each delimiting a separate target region, are known in the art. It is most common in such reactions for each target region to amplify less well than

if each target region were amplified in separate reactions. Not only do both (or all) true-target amplicons compete with each other for amplification reaction components, but the potential for non-target initiation and competitive amplification should increase as (about) $p_p \times p_t$, where p_p is the total concentration of all promoter-primers in the reaction and p_t is the total concentration of all primers present (or $-p_t^2$ in a case such as routine PCR wherein all the primers are functionally equivalent for initiation).

These complications are a significant impediment to routine development of multiplex amplification systems with reliable detection sensitivity for very low template levels. Nevertheless, using the target-specific initiation enhancements described herein, we have been successful in identifying a multiplex reaction composition with high sensitivity for both *pol1* and *gag11* targets.

Table 7 summarizes the results of one such experiment. Condition A was a standard IM2 procedure in which each of the ten (10) replicate reactions received the *pol1* inside primers (T74116 and 4195) and the *gag11* primers (T7811 and 872) but no outside primers. After amplification, 50 μ l of each reaction was removed and analyzed by hybridization using the *pol1* probe. The remaining 50 μ l of each reaction was analyzed using the *gag11* probe. The results in the *pol1* section of column A are arrayed in the same sample order as the *gag11* results. (i.e., 50 μ l of sample #1 yielded 7,448 RLU when analyzed with the *pol1* probe; the remaining 50 μ l gave 19,596 RLU when analyzed with the *gag11* probe.)

Likewise, the RLU values in column C reflect analysis of half of each respective reaction using the *pol1* or *gag11* probes as shown. Condition C was the enhanced initiation procedure described under General Methods above except that eight oligonucleotide primers were present (780, T7811, 872, T71062, 4009, T74116, 4195 and T74312,

at 5, 30, 30, 10, 3, 15, 30 and 3 pmol/reaction, respectively).

Condition B was the enhanced initiation procedure using only the four pol1 primers, and condition D samples received only the four gag11 primers. Each of the replicate reactions in B and D was analyzed by hybridization using the full reaction volume.

The reactions shown in Table 7 each received an average of 5 pUCHIV templates and 50 μ l of lysate prepared as described in Example 4. The corresponding negative controls for these conditions (A, B, C, A', D, and C') were 2094, 2907, 2925, 1799, 2014 and 2315, respectively.

Table 7

	Multiplex IM2	Separate Enhanced	Multiplex Enhanced
	A	B	C
pol1	7,448	1,150,134	1,078,254
	3,397	1,201,876	1,143,278
	3,314	1,170,546	1,106,627
	3,469	1,160,177	1,112,210
	3,314	1,143,588	1,111,058
	3,226	1,154,678	1,140,999
	3,136	1,153,301	1,118,935
	3,192	1,195,168	3,120
	3,185	1,178,318	1,136,254
	151,392	1,204,093	1,122,474
G.M.:	5,220	1,170,994	621,254
	A'	D	C'
gag11	19,596	389,763	81,091
	2,009	327,397	92,182
	17,717	212,354	110,175
	2,386	318,371	107,628
	2,065	345,542	74,106
	211,008	280,156	78,950
	26,975	120,927	173,221
	84,759	323,234	2,129
	69,965	167,985	76,044
	145,029	162,030	68,690
G.M.:	21,018	248,238	63,089

It was apparent from the gag11 results with the enhanced system that this target region amplified to a greater extent in a reaction comprising only gag11 primers (D) than in a reaction comprising pol1 and gag11 primers (C'). Furthermore, it was apparent for both target regions, especially pol1, that detection effectiveness was significantly greater in the enhanced multiplex system (C) than for the multiplex IM2 system (A). Note that the superior initiation effectiveness of gag11 (A') compared to pol1 (A) in standard IM2 is consistent with many previous results.

Baseline signal levels (pol1 RLU=3120, gag11 RLU=2129) were observed in the same enhanced multiplex samples (C,C') when analyzed by hybridization with each probe, indicating that there was no HIV DNA in these samples to be amplified. A single failure in 10 replicates is not unexpected at the 5 template input level based on the Poisson distribution ($p \geq 0.065$). Therefore, these results indicate that this multiplex system is able to detect single copies of two different target regions in the same reaction.

Other embodiments are within the following claims.

SEQUENCE LISTING

- 1) GENERAL INFORMATION:
- (i) APPLICANT: Thomas B. Ryder, Elizabeth R. Billyard and Nanibushan Dattagupta
 - (ii) TITLE OF INVENTION: NUCLEIC ACID AMPLIFICATION
 - (iii) NUMBER OF SEQUENCES: 8
 - (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Lyon & Lyon
 - (B) STREET: 611 West Sixth Street,
Suite 3400
 - (C) CITY: Los Angeles
 - (D) STATE: California
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 90017
 - (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb storage
 - (B) COMPUTER: MacIntosh Powerbook 140
 - (C) OPERATING SYSTEM: Apple P.C. DOS (version)
 - (D) SOFTWARE: Microsoft Word (Version)
 - 5.0) (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Warburg, Richard J.
 - (B) REGISTRATION NUMBER: 32,327
 - (C) REFERENCE/DOCKET NUMBER:
 - (ix) TELECOMMUNICATION INFORMATION:

39

(A) TELEPHONE: (213) 489-1600
(B) TELEFAX: (213) 955-0440
(C) TELEX: 67-3510

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: nucleic
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATTCCCTACA ATCCCCAAAG TCAA

24

2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48
(B) TYPE: nucleic
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 2

AATTTAATAC GACTCACTAT AGGGAGACAA ATGGCAGTAT TCATCCACA

39

2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23
(B) TYPE: nucleic
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 3

GTTTGTATGT CTGTTGCTAT TAT

23

2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45
(B) TYPE: nucleic
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 4

AATTTAATAC GACTCACTAT AGGGAGACCC TTCACCTTTC CAGAG

45

2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25
- (B) TYPE: nucleic
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 5

TGCACCAGGC CAGATGAGAG AACCA

25

2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 46
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 6

AATTTAATAC GACTCACTAT AGGGAGAAGT GACATAGCAG GAACTA

46

2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 7

AGATTTCTCC TACTGGGATA GGT

33

2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 8

AATTTAATAC GACTCACTAT AGGGAGATTG GACCAGCAAG GTTTCTGTC

49

Claims

1. Method for amplification of a nucleic acid strand in a test sample, comprising the steps of:

first contacting said nucleic acid strand from said
5 test sample simultaneously with at least three oligonucleotide primers, at least one primer being a promoter-primer, at least one said primer being complementary to said nucleic acid strand, and at least one other said primer being complementary to a strand complementary to
10 said nucleic acid strand; and

second contacting said nucleic acid strand and primers with one or more proteins having RNA-directed and/or DNA-directed DNA polymerase activities, an RNA polymerase activity and an RNase H activity, under primer-extension
15 conditions to allow amplification of said nucleic acid strand at constant temperature.

2. The method of claim 1, wherein said nucleic acid strand is a DNA strand, and prior to said second contacting step said nucleic acid strand and primers are contact-
20 ed at about 60°C or above with an enzyme having DNA polymerase activity active at about 60°C or above.

3. The method of claim 1, wherein said second contacting step is at about 42°C or above in the presence of a reverse transcriptase.

25 4. The method of claim 1 wherein said nucleic acid strand and said primers are heated to about 95°C or higher prior to said second contacting step.

5. The method of any claims 1-4 wherein four primers are used in said first contacting step.

30 6. The method of claim 1, wherein one said primer is provided at a concentration different from one other said primer.

7. The method of claim 5, wherein one said primer is provided at a concentration different from one other said primer.
8. The method of claim 5, wherein two said primers
5 are provided at a concentration different from said other primers.
9. The method of claim 1, wherein two said primers are plus-sense primers and the inside plus-sense primer is a promoter-primer.
- 10 10. The method of claim 1 wherein two said primers are minus-sense primers and said outside primer is a promoter-primer.
11. The method of claim 1 wherein all said enzyme
15 activities are provided by a reverse transcriptase and an RNA polymerase.
12. The method of claim 11 wherein said enzyme activities are supplemented by an RNase H having no DNA polymerase activity.
13. The method of claim 2 wherein said DNA polymerase
20 lacks 5'-3' exonuclease activity.
14. The method of claim 13 wherein said DNA polymerase is derived from a DNA polymerase, which in its natural form possesses 5'-3' exonuclease activity.
15. The method of claim 13 or 14, wherein said DNA
25 polymerase is the DNA polymerase I of a Bacillus species.
16. The method of claim 15, wherein said species is Bacillus stearothermophilus or Bacillus caldotenax.

17. The method of claim 1 wherein the two outside primers hybridize to said nucleic acid strand or its complement at most 2000 bases apart.

18. The method of claim 1 wherein the two outside
5 primers hybridize to said nucleic acid strand or its complement at most 500 bases apart.

19. The method of claim 1 wherein the two outside primers hybridize to said nucleic acid strand or its complement at most 350 bases apart.

10 20. The method of claim 6 wherein one said primer is provided at a concentration between 1 and 10 μM and another said primer is provided at a concentration between 10 and 50 μM .

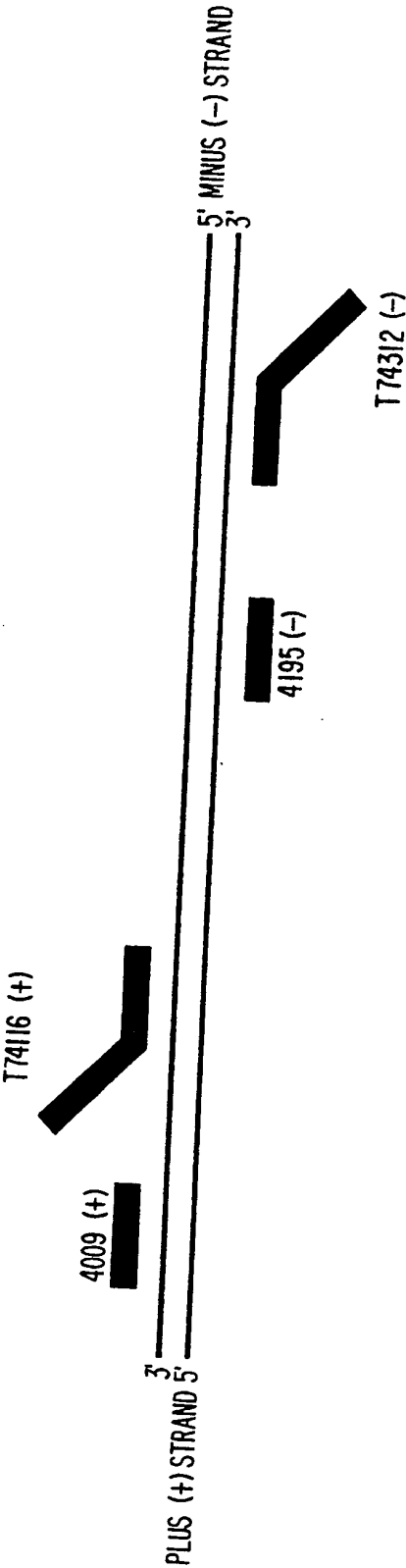


FIG. 1.

FIG. 2.

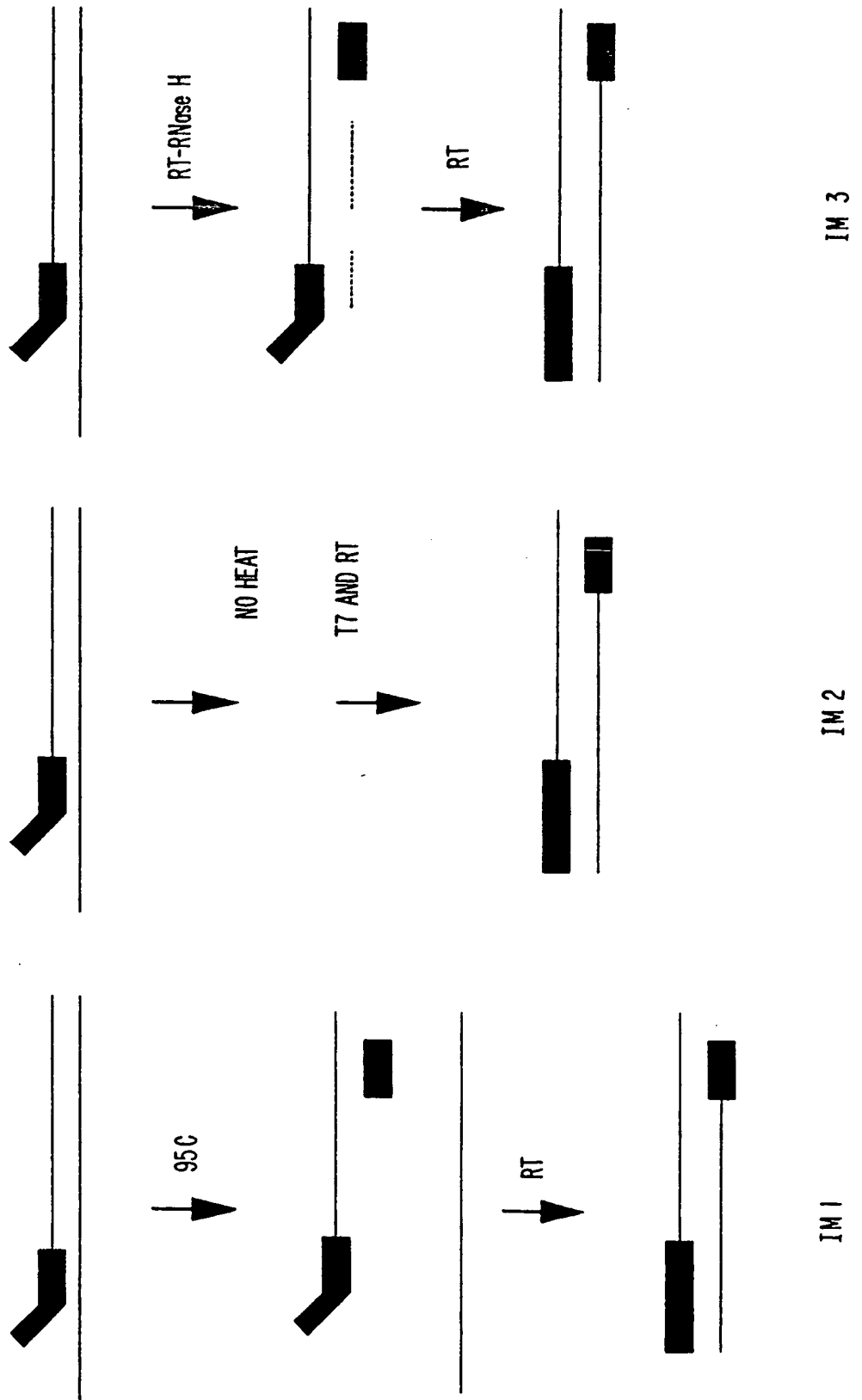
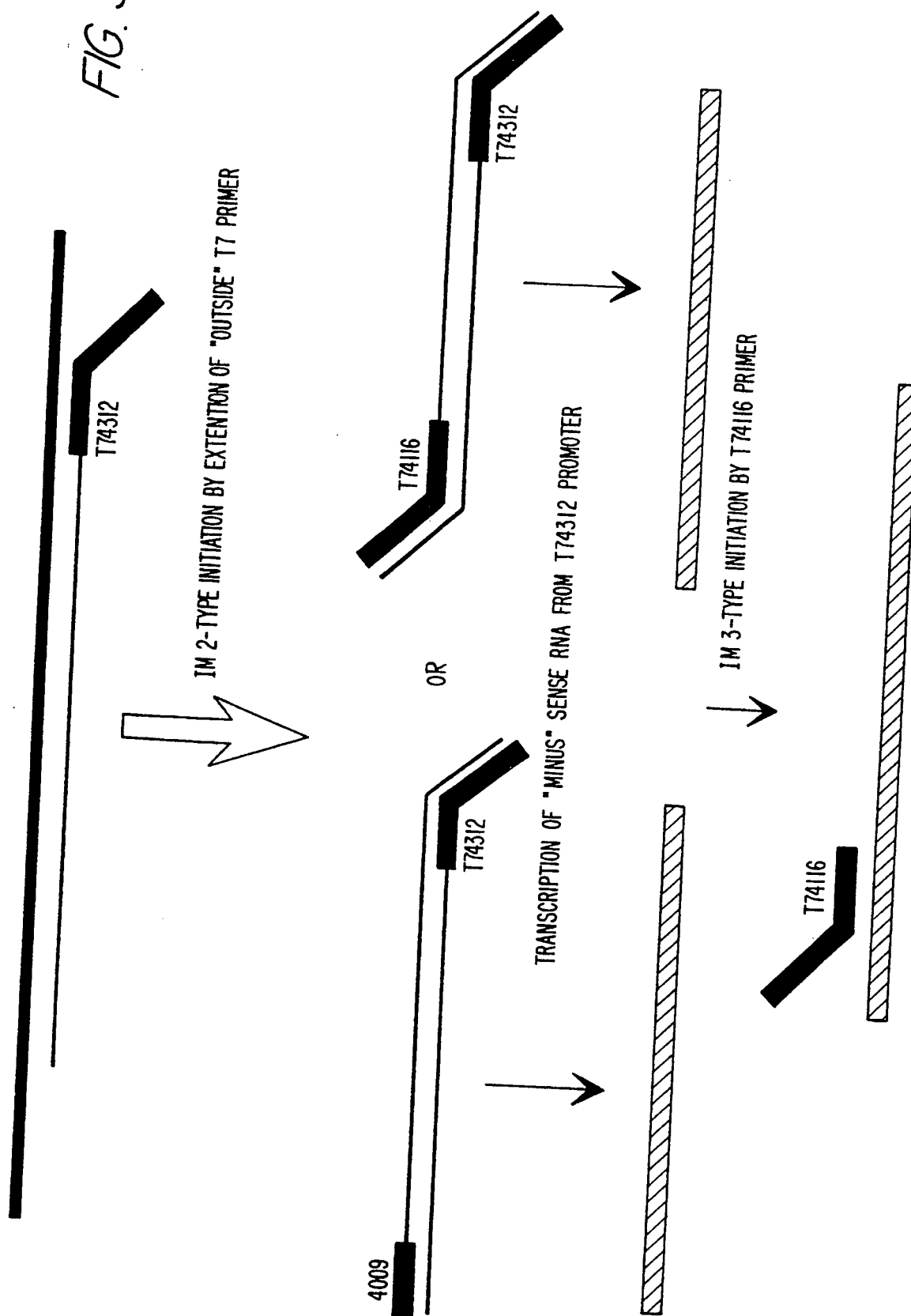


FIG. 3



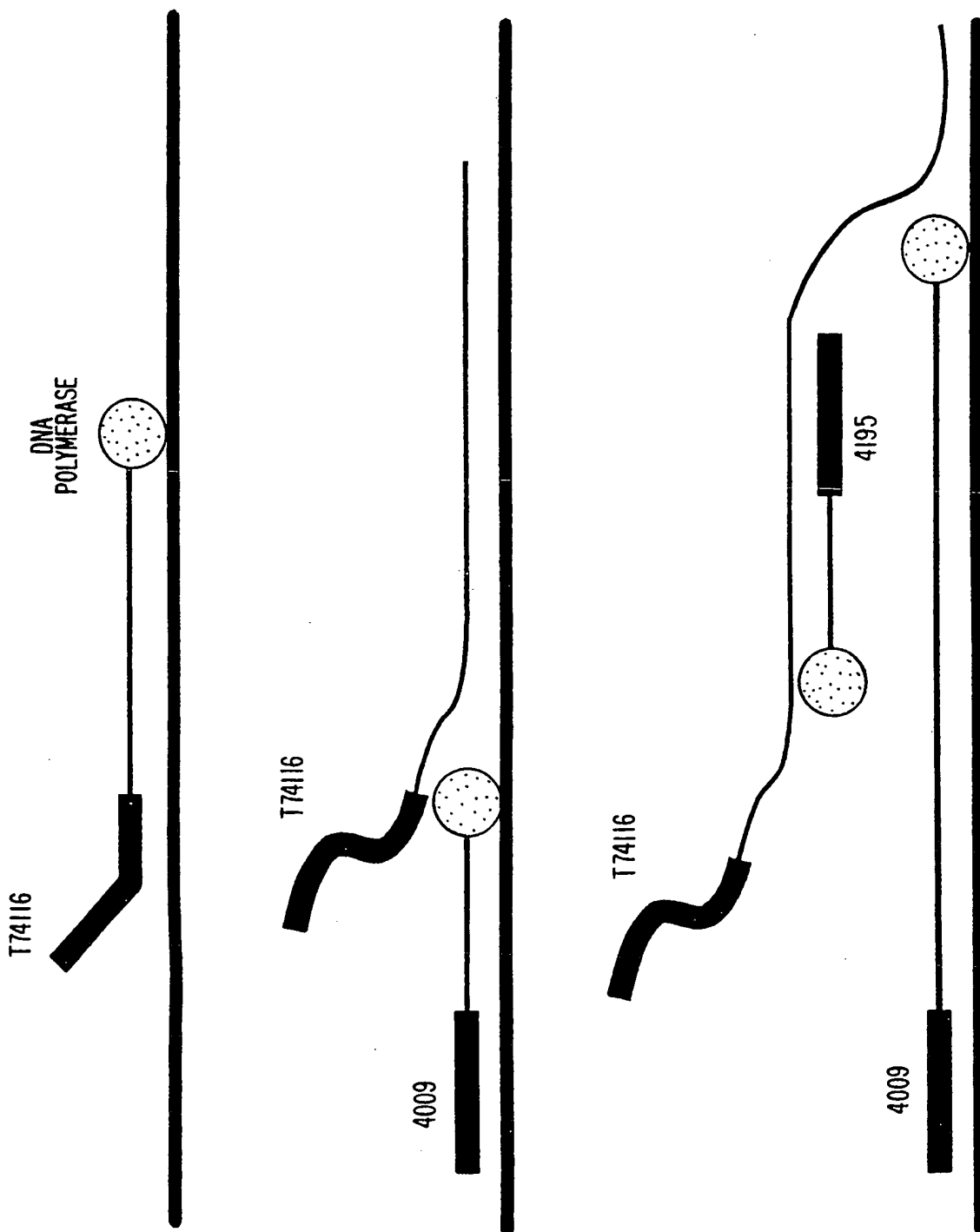


FIG. 4.

INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 94/08307

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF VIROLOGICAL METHODS, vol.35, 1991, AMSTERDAM NL pages 273 - 286 T.KIEVITS ET AL. cited in the application see page 275; figure 1 see page 280, paragraph 2 ---	1,3-5
X	EP,A,0 545 010 (BOEHRINGER MANNHEIM GMBH) 9 June 1993 see page 5, line 7 - line 48 see page 6, line 13 - line 50 --- -/--	1,3,4,6, 11,12, 17-20

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

9 November 1994

Date of mailing of the international search report

02.01.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+ 31-70) 340-3016

Authorized officer

De Kok, A

INTERNATIONAL SEARCH REPORT

Internati Application No

PCT/US 94/08307

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 408 295 (GENE-PROBE INCORPORATED) 16 January 1991 cited in the application see page 1 - page 18	1
A	---	2-4,9-12
Y	SCIENCE, vol.252, no.5013, 21 June 1991, LANCASTER, PA US pages 1643 - 1651 H.A.ERLICH ET AL. see page 1643 - page 1645	1
A	---	2-10
A	EP,A,0 519 338 (F. HOFFMANN-LA ROCHE AG) 23 December 1992 see page 3, line 7 - line 25 see page 4, line 5 - line 55 see page 5, line 2 - line 30 see page 8, line 11 - line 43 see page 9, line 12 - line 18 ---	1-6, 9-13, 17-19
A	DNA AND CELL BIOLOGY, vol.10, no.3, 1991, NEW YORK US pages 233 - 238, XP444410 D.Y. WU ET AL. see the whole document ---	2
A	EP,A,0 469 610 (SHIONOGI SEIYAKU KABUSHIKI KAISHA) 5 February 1992 see page 1, line 31 - page 2, line 56 ---	1,2,4-8, 17-20
A	EP,A,0 329 822 (CANGENE CORPORATION) 30 August 1989 see the whole document ---	1-4, 11-14
A	JOURNAL OF BACTERIOLOGY, vol.174, no.13, 1992, BALTIMORE US pages 4350 - 4355 E.SELLMANN ET AL. see the whole document ---	13-16
A	AMERICAN BIOTECHNOLOGY LABORATORY, vol.10, no.9, 1992, NEW YORK US page 47 Y.ISHINO see the whole document ---	13-16
P,X	ANALYTICAL BIOCHEMISTRY, vol.217, no.2, March 1994, ORLANDO US pages 248 - 254 T.LIVACHE ET AL. see the whole document -----	1,2,4,5, 9,10

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat Application No

PCT/US 94/08307

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0545010	09-06-93	DE-A- 4213029	01-04-93
		JP-A- 5244951	24-09-93
EP-A-0408295	16-01-91	AU-B- 650622	30-06-94
		AU-A- 6166390	22-02-91
		CA-A- 2020958	12-01-91
		JP-T- 4500759	13-02-92
		WO-A- 9101384	07-02-91
EP-A-0519338	23-12-92	CA-A- 2071594	21-12-92
		JP-A- 5292968	09-11-93
		US-A- 5314809	24-05-94
EP-A-0469610	05-02-92	JP-A- 4091790	25-03-92
EP-A-0329822	30-08-89	AT-T- 106948	15-06-94
		DE-D- 3850093	14-07-94
		DE-T- 3850093	03-11-94
		WO-A- 9102814	07-03-91
		ES-T- 2053648	01-08-94
		JP-A- 2005864	10-01-90

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☐ **BLACK BORDERS**

☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**

☐ **FADED TEXT OR DRAWING**

☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**

☒ **SKewed/SLANTED IMAGES**

☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**

☐ **GRAY SCALE DOCUMENTS**

☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**

☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**

☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)